

# Exhibit 9

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## Inhibition of the Cardiac p38-MAPK Pathway by SB203580 Delays Ischemic Cell Death

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**Summary:** We report that SB203580 (SB), a specific inhibitor of p38-MAPK, protects pig myocardium against ischemic injury in an in vivo model. SB was applied by local infusion into the subsequently ischemic myocardium for 60 min before a 60-min period of coronary occlusion followed by 60-min reperfusion (index ischemia). Infarct size was reduced from a control value of  $69.3 \pm 2.7\%$  to  $36.8 \pm 3.7\%$ . When SB was infused systemically for 10 min before index ischemia, infarct size was reduced to  $36.1 \pm 5.6\%$ . We measured the content of phosphorylated p38-MAPK after systemic infusion of SB and Krebs-Henseleit buffer (KHB; negative control) and during the subsequent ischemic period using an antibody that reacts specifically with dual-phosphorylated p38-MAPK (Thr180/Tyr182). Ischemia with and without SB significantly increased phospho-p38-MAPK, with a maximum reached at 20 min but was less at 30 and 45 min under the influence of the inhibitor. The systemic infusion of SB for 10 min before index ischemia did not significantly change the p38-MAPK activities (compared with vehicle, studied by in-gel phosphorylation)  $\leq 20$  min

of ischemia, but activities were reduced at 30 and 45 min. Measurements of p38-MAPK activities in situations in which SB was present during in-gel phosphorylation showed significant inhibition of p38-MAPK activities. The systemic infusion of SB significantly inhibited the ischemia-induced phosphorylation of nuclear activating transcription factor 2 (ATF-2). Using a specific ATF-2 antibody, we did not observe significant changes in ATF-2 abundance when nuclear fractions from untreated, KHB-, and SB-treated tissues were compared. We investigated also the effect of local and systemic infusion of SB on the cardioprotection induced by ischemic preconditioning (IP). The infusions (local or systemic) of SB before and during the IP protocol did not influence the infarct size reduction mediated by IP. The observed protection of the myocardium against ischemic damage by SB points to the negative role of the p38-MAPK pathway during ischemia. **Key Words:** SB203580—Protein kinases—p38-MAPK—Ischemia/reperfusion—Pig.

Stressful stimuli applied as brief pulse trains condition most tissues so that they become more tolerant against longer lasting stresses, significantly delaying the onset of irreversible damage (1). The molecular mechanism of this increase in stress tolerance, especially that toward ischemia, is not entirely clear, but mitogen activated protein kinases (MAPKs), which are involved in the signal-transduction pathways, may play a role. In previous reports we have shown that brief ischemic pulses lead to changes in the expression of the cardiac protooncogenes that may participate in the adaptive response (2,3). Since the protooncogene-based transcription factors are activated by membrane and cytoplasmic signaling cascades, we studied the involvement of three MAPK pathways

(extracellular signal-regulated kinases, ERKs; stress-activated/c-Jun N-terminal kinases, SAPK/JNKs; and p38-MAPK) and found that they react to brief ischemia and reperfusion in a very specific way: the ERKs moderately increased activity during brief ischemia but markedly during reperfusion, the SAPK/JNKs become active only during reperfusion, and the p38-MAPK was activated only during ischemia and deactivated during the following reperfusion and subsequent period of ischemia (4). MAPK pathways can also be influenced by pharmacologic agents that specifically influence the activity of members of the MAPKs, particularly those of the stress-activated protein kinases (SAPK/JNKs, p38-MAPK) (5,6). A common feature of all MAPKs is their

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ability to phosphorylate the transactivation domains of numerous transcription factors and thus modulate transcriptional activity. However, it cannot be excluded that MAPK activation also has effectors outside the nucleus. The fact that repeated brief occlusion, which can amplify the conditioning effect of the first occlusion, induced an attenuation of p38-MAPK activity suggested that p38-MAPK activation may cause premature ischemic cell death. We hypothesized that SB203580 (SB), an inhibitor of p38-MAPK, may be able to protect the heart against the consequences of prolonged ischemia. This hypothesis was tested in an in vivo model by two different application methods of SB: intramyocardial and intravenous infusion.

## MATERIALS AND METHODS

The experimental protocol described in this study was approved by the Bioethical Committee of the District of Darmstadt, Germany. Furthermore, all animals in this study were handled in accordance with the guiding principles in care and use of animals as approved by the American Physiology Society, and the investigation conformed with the Guide for Care and Use of Laboratory Animals published by the U.S. National Institutes of Health.

### Chemicals

Azaperone, metomidate, and piritramide were purchased from Janssen Pharmaceutica (Meckenheim, Germany). SB, protein kinase inhibitor (PKI),  $\alpha$ -chloralose, triphenyl tetrazolium chloride (TTC), and other biochemicals were from Sigma (Deisenhofen, Germany). The fluorescent zinc-cadmium sulfide microspheres (diameter, 2–15  $\mu$ m) were purchased from Duke Scientific Corp (AC Leusden, Netherlands). The polyclonal antibody against p38-MAPK was from Santa Cruz Biotechnology (Heidelberg, Germany). Phospho-p38-MAPK, phospho-SAPK/JNK, ATF-2, and phospho-ATF-2 antibodies were from New England Biolabs (Schwalbach/Taunus, Germany). Nitrocellulose membranes, rainbow molecular mass markers, the horseradish peroxidase-linked goat anti-rabbit immunoglobulin, the enhanced chemiluminescence (ECL) reagents, autoradiography films, and [ $\gamma$ - $^{32}$ P]-ATP were from Amersham (Pharmacia Biotech, Europe GmbH, Freiburg, Germany). Recombinant MAPKAPK-2 (residues 46–400 encompassing the catalytic domain) was expressed in *Escherichia coli* as glutathione-S-transferase fusion protein (clone provided by C. J. Marshall, a kind gift from P. H. Sugden) and was purified by glutathione-Sepharose (Pharmacia) chromatography.

### Animal preparation

Male castrated German landrace-type domestic pigs (32.6  $\pm$  2.3 kg) were premedicated with azaperone (2 mg/kg of body weight, i.m.) and 2 mg/kg BW piritramide, s.c., 30 min before the initiation of anesthesia with 10 mg/kg BW metomidate. After tracheal intubation, a bolus of  $\alpha$ -chloralose (25 mg/kg) was given intravenously. Anesthesia was maintained by a continuous intravenous infusion of  $\alpha$ -chloralose (25 mg/kg/h). The animals were ventilated artificially with a pressure-controlled respirator (Stephan Respirator ABV, F. Stephan GmbH, Gackebach, Germany) with room air enriched with 2 L/min of oxygen. Arterial blood gases were analyzed frequently to guide

adjustment of the respirator settings. Additional doses of piritramide (10 mg) were given i.v. every 60 min. Both internal jugular veins were cannulated with polyethylene tubes for administration of saline, piritramide, and  $\alpha$ -chloralose. Arterial sheath catheters (7F) were inserted into both carotid arteries. To measure arterial blood pressure, the left sheath was advanced into the aortic arch and connected with a Statham transducer (P23XL; Statham, San Juan, Puerto Rico). After a midsternal thoracotomy, the heart was suspended in a pericardial cradle. Arterial pressure, heart rate, and the ECG were continuously monitored and recorded on the hard disk of a MacLab computer. A loose reversible ligature was placed halfway around the left anterior descending artery (LAD), and was subsequently tightened to occlude the vessels. In pigs subjected to intramyocardial microinfusion, eight 26-gauge needles connected by tubing with a peristaltic pump (Miniplus; Gilson, Villiers-le-Bel, France) were placed in pairs along the LAD into the myocardium perpendicular to the epicardial surface. After preparation, a stabilization period of 30 min was allowed and the experimental protocols were started. The p38-MAPK inhibitor, SB, was dissolved in DMSO and finally diluted in Krebs-Henseleit buffer (KHB; final concentration of DMSO was 0.1%). For this reason, the infusion of KHB with DMSO served as a negative control (KHB).

### Experimental groups

This study consisted of eight experimental groups (Fig. 1). Group I was subjected to 60 min of occlusion and 60 min of reperfusion (control group 1). In group II, SB (40 nM) or KHB (with 0.1% DMSO) was administered by local infusion for 60 min before the index ischemia of 60 min and the following reperfusion period of 60 min. In group III, SB (5 mg/animal) or KHB was applied by systemic infusion for 10 min before the index ischemia (60 min occlusion and 60 min reperfusion periods). In group IV, the animals were subjected to 40 min of occlusion followed by 60 min of reperfusion (control group 2). In group V, the animals were subjected to the preconditioning protocol (two cycles of 10-min ischemia and 10-min reperfusion) followed by a period of 40-min index ischemia and 60 min of reperfusion. In group VI, SB (40 nM) or KHB was administered by local microinfusion for 15 min before the brief occlusions/reperfusions and during reperfusion periods of the preconditioning protocol. This was followed by 40 min of ischemia and 60 min of reperfusion. In group VII, SB (5 mg/animal) or KHB was applied by intravenous infusion for 15 min before the brief occlusions/reperfusions and during reperfusion periods of the preconditioning protocol. This was followed by 40 min of index ischemia and 60 min of reperfusion. In group VIII, SB (5 mg/animal) or KHB was applied by intravenous infusion for 10 min before the index ischemia of 60 min, and left ventricular biopsies for in vitro assays were obtained at the end of SB and KHB infusion and at 5, 10, 20, 30, 45, and 60 min of the following index ischemia. Drill biopsies were taken from control tissue, KHB-, and SB-treated tissue (Fig. 1). Biopsies weighed ~80 mg and were ~4 mm long (i.e., they reached from epi- to midmyocardium).

### Determination of infarct size

At 45 min into the last reperfusion period, 1 g of fluorescein dissolved in 10 ml Ringer's solution was injected into the right ventricle. This stained the entire myocardium and detected non-reperfused tissue. Hearts with traces of nonreperfused myocardium were excluded from analysis. At the end of the experi-

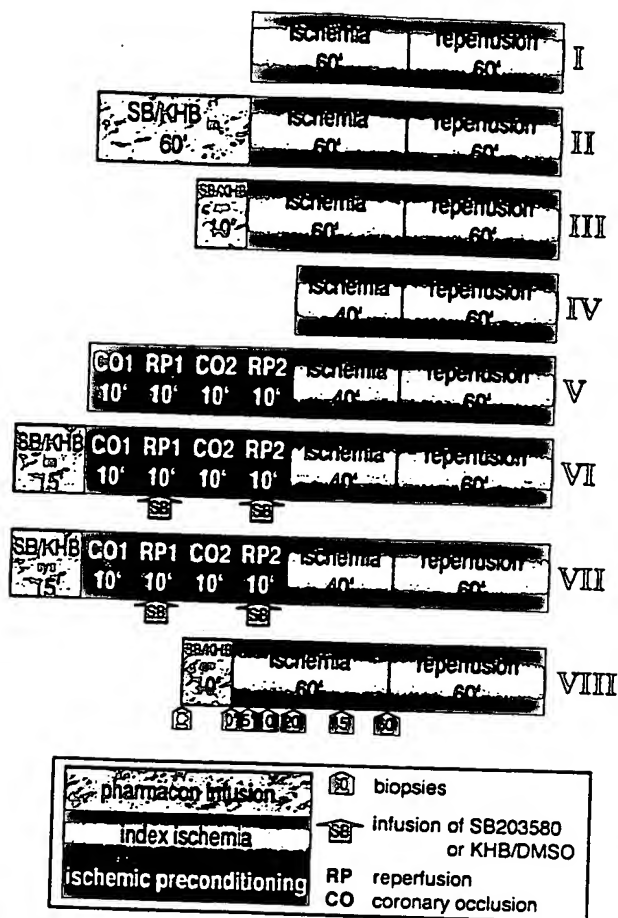


FIG. 1. This study consisted of eight experimental groups. Group I was subjected to 60 min of occlusion and 60 min of reperfusion (control group 1). In group II, SB203580 (40 nM) or KHB (with 0.1% DMSO, negative control) were administered by local intramyocardial infusion (i.m.; infusion rate, 20  $\mu$ l/min) for 60 min before the index ischemia of 60 min and the following reperfusion period of 60 min. In group III, SB203580 (5 mg/animal) or KHB was applied by systemic infusion (sys) for 10 min before the index ischemia of 60 min and the following reperfusion period of 60 min. In group IV, the animals were subjected to 40 min of occlusion followed by 60 min of reperfusion (control group 2). In group V, the animals were subjected to a preconditioning protocol of two cycles of 10-min ischemia and 10-min reperfusion followed by the index ischemia of 40-min occlusion and a reperfusion period of 60 min. In group VI, SB203580 (40 nM) or KHB was administered by local microinfusion for 15 min before preconditioning and during the reperfusion phase of the preconditioning protocol. This was followed by index ischemia of 40-min occlusion and a reperfusion period of 60 min. In group VII, SB203580 (5 mg/animal) or KHB was applied by systemic infusion for 15 min before preconditioning and during the reperfusion phases. This was followed by the index ischemia of 40-min occlusion and the reperfusion period of 60 min. In group VIII, SB203580 (5 mg/animal) or KHB was systematically infused for 10 min before index ischemia of 60 min, and left ventricular biopsies for *in vitro* assays were obtained at the end of SB and KHB infusion and at various intervals of index ischemia.

mental protocol, the LAD and the aorta were occluded, clamped, and 500 mg of zinc cadmium fluorescent microspheres in 10 ml of Ringer's solution were injected into the ascending aorta. Shortly thereafter, the animals were injected

with an intravenous bolus of 20% potassium chloride to arrest the heart. After excision, both atria and the right ventricle were removed. The left ventricle was cut into slices along the microinfusion needle pairs perpendicular to the LAD. Heart slices were weighed and incubated at 37°C in 1% triphenyltetrazolium chloride (TTC) in PBS, pH 7.0, for 20 min. Myocardium at risk of infarction was identified as the nonfluorescent (by microspheres) area by UV light (366 nm). The infarcted area was demarcated by the absence of the characteristic red TTC stain. The slices were photographed by double exposure with UV and artificial daylight, and the pictures were used for further planimetric evaluation. Planimetry of the infarct areas was performed on the basal aspect of the apex, the apical and basal sides of the following four consecutive myocardial slices, and on the apical aspect of the basal section of the left ventricle. We expressed infarct size (IS) as the infarct area (IA) relative to the risk area (RA). Infarct sizes were then averaged per group and depicted graphically (Figs. 3 and 5).

#### Preparation of soluble and nuclear fractions

The biopsies for the kinase assays were suspended in ice-cold buffer containing in mM: 20 Tris-HCl, 250 sucrose, 1.0 EDTA, 1.0 EGTA, 1.0 dithiothreitol (DTT), 0.1 sodium orthovanadate, 10 NaF, and 0.5 PMSF, pH 7.4, (buffer A), and were homogenized with a Teflon-glass homogenizer. The homogenates were centrifuged at 14,000 g for 30 min at 4°C. The supernatants represented the soluble (cytosolic) fractions. The pellets were resuspended in buffer B containing in mM: 20 Tris-HCl, 1,000 sucrose, 1.0 EDTA, 1.0 EGTA, 1.0 DTT, 0.1 sodium orthovanadate, 10 NaF, 10 KCl, and 0.1 PMSF (pH 7.4), and were centrifuged for 30 min at 10,000 g (4°C). The resulting pellets were resuspended in buffer C containing 10% glycerol, 20 mM Tris-HCl, 400 mM KCl, 1.0 mM EGTA, 1.0 mM DTT, 0.1 mM sodium orthovanadate, 10 mM NaF, 0.5 mM PMSF, and 0.1% Triton X-100, and sonicated and used for the detection of transcription factor ATF-2. For the preparation of electrophoretic probes, Laemmli sample buffer was added and the proteins were denatured by heating. The denatured probes were applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and used for MAPK assays by *in-gel* kinase assays and for Western blot analysis.

#### Measurement of p38-MAPK activities by *in-gel* phosphorylation

Equivalent amounts of proteins were separated on 10% SDS-polyacrylamide gels containing 0.5 mg/ml of GST-MAPKAPK-2<sub>46-400</sub>. After electrophoresis, the gels were washed for 1 h with 20% (vol/vol) 2-propanol in 50 mM Tris-HCl (pH 8.0), followed by 1 h with 5 mM 2-mercaptoethanol in 50 mM Tris-HCl, pH 8.0. The *in-gel* proteins were denatured by incubation for 2 h with 50 mM Tris-HCl, pH 8.0, containing 6 M guanidine-HCl. Renaturation was achieved by incubation with 50 mM Tris-HCl, pH 8.0, containing 0.1% (vol/vol) Nonidet P-40 and 5 mM 2-mercaptoethanol for 16 h. After preincubation of gels in 40 mM HEPES (pH 8.0) containing 2 mM DTT and 10 mM magnesium chloride, the *in-gel* phosphorylation of substrates was performed in 40 mM HEPES (pH 8.0), 0.5 mM EGTA, 10 mM magnesium chloride, 1.0  $\mu$ M protein kinase A inhibitory peptide, and 25  $\mu$ M [ $\gamma$ -<sup>32</sup>P]-ATP (5  $\mu$ Ci/ml) at 25°C for 4 h. In some experiments, the sample preparation, incubation of gels, and phosphorylation were performed in the presence of 50 nM SB. After extensive washing in 5% (wt/vol) trichloroacetic acid containing 2% (wt/vol) sodium pyrophosphate, the gels were dried, and quantitative analysis was

performed using a Phosphorimager SF (Molecular Dynamics, Krefeld, Germany).

### Immunoblot analysis

Soluble or nuclear fractions of heart were subjected to SDS-PAGE in 10% polyacrylamide gels, and proteins were transferred onto nitrocellulose membranes. Anti-p38-MAPK, anti-phospho-p38-MAPK, anti-phospho-SAPK/JNK, anti-ATF-2, and anti-phospho-ATF-2 antibodies were used for primary immunodetection. The secondary antibody directed against all antibodies was peroxidase-labeled anti-rabbit immunoglobulin. Bound antibodies were detected by the ECL Western blot detection method.

### Statistics

For the IS quantification, we used the unpaired Student's *t* test;  $p < 0.05$  was accepted as significant. For in-gel phosphorylation and Western blot assays, the SB-treated tissue biopsy material was compared with control (untreated) and KHB-treated tissue (negative control). The differences were evaluated with a Student's *t* test. The accepted level of significance was  $p < 0.05$ .

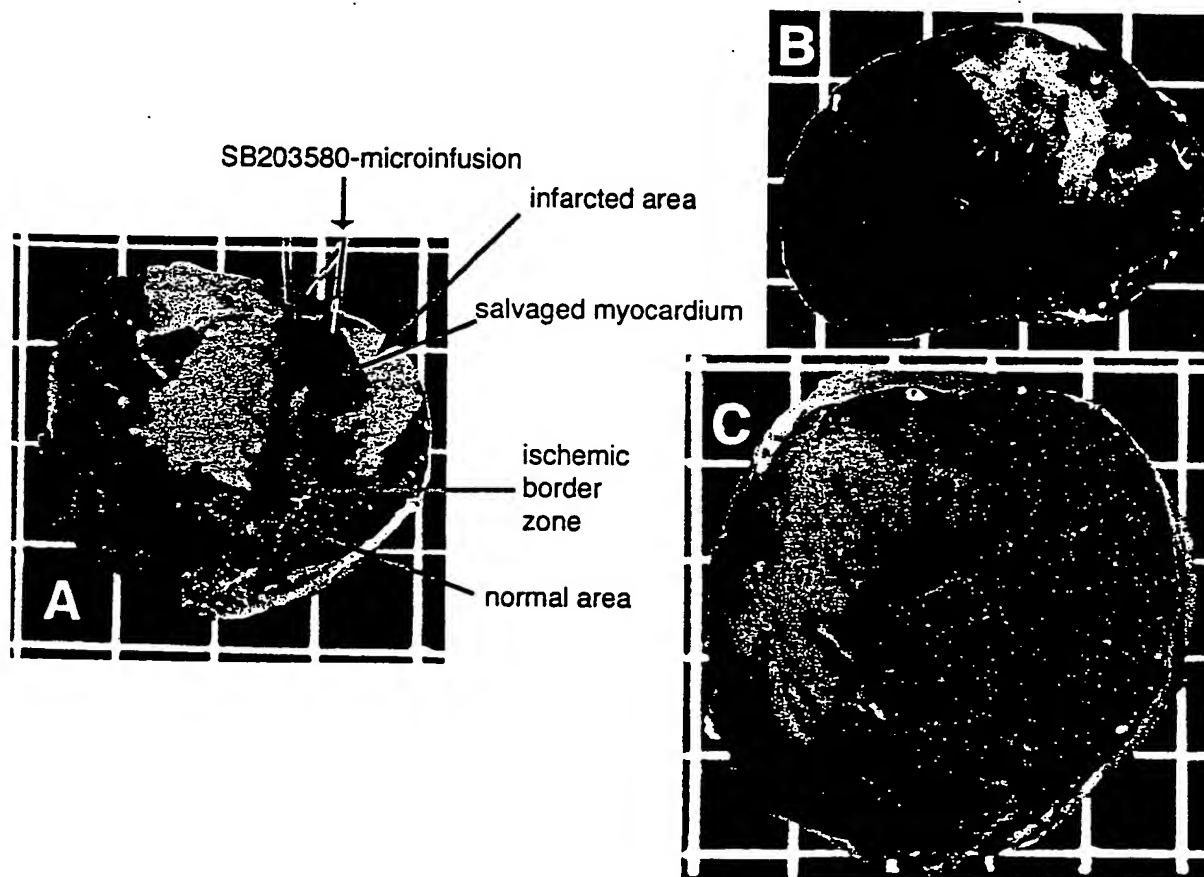
## RESULTS

### Hemodynamic data

The infusion of SB, systemic and local, had no effect on blood pressure and heart rate. Coronary occlusion produced a decrease in blood pressure that usually returned to normal values before reperfusion.

### The effect of SB203580 infusion on infarct size

The effects of local and systemic infusions of the p38-MAPK inhibitor SB on IS in pig myocardium are shown in Figs. 2 and 3. The local intramyocardial infusion of SB203580 (40 nM) for 60 min before index ischemia (group II) significantly reduced infarct size from  $69.3 \pm 2.7\%$  (control, group I) to  $36.8 \pm 3.7\%$  ( $p < 0.002$ ; Fig. 3). When SB was infused intravenously (5 mg/animal) for 10 min before the onset of 60-min coronary occlusion (group III), we also observed a significant reduction of IS as compared with control (group I;  $36.1 \pm 5.6\%$  for SB,  $69.3 \pm 2.7\%$  for control). The remaining infarcts were not solid but rather spotty. Important also is the fact that both local and systemic infusions of KHB/DMSO (0.1%



**FIG. 2.** Intramyocardial infusion of SB203580 (group II; A) and systemic infusion of SB203580 (group III; B) before index ischemia of 60-min occlusion followed by reperfusion of 60 min. The needles for intramyocardial microinfusion were placed into the subsequently ischemic part of the left ventricle. The fluorescent microspheres demarcate the nonfluorescent area of risk. After TTC staining, the myocardial protection was defined as stained tissue surrounding the microinfusion needles in transmurally infarcted myocardium. A: Microinfusion of SB203580 (40 nM, needle on the right). B: Systemic infusion of SB203580 (5 mg/animal). C: Index ischemia for 60-min occlusion followed by reperfusion of 60 min (control group 1).

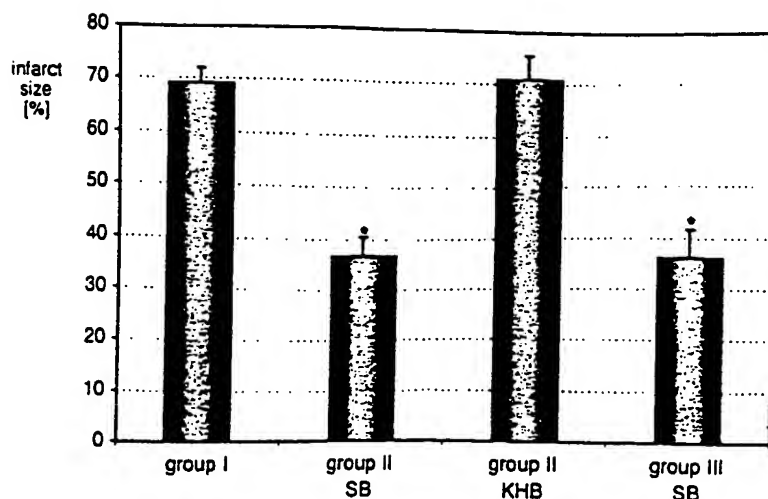


FIG. 3. Effect of local intramyocardial (group II) and systemic infusion (group III) of SB203580 before index ischemia on infarct size in pig myocardium. Group II: Effect of KHB/DMSO infusion on infarct size. Values are expressed as percentage of the area at risk of infarction (group I, control group 1). Each bar represents the mean  $\pm$  SEM. \* $p < 0.002$  (vs. group I).

DMSO in KHB, negative control) before index ischemia did not influence the IS as compared with control (Fig. 3).

#### The effect of SB203580 on ischemic preconditioning

The effect of local and systemic infusion of SB on cardioprotection by ischemic preconditioning is shown in Figs. 4 and 5. When SB was applied locally before and during the ischemic preconditioning protocol (group VI), the IS represented  $3.8 \pm 0.5\%$ . This IS was significantly lower than that in control 2 (group IV; IS,  $54.0 \pm 2.5\%$ ) and was not different from group V (IS,  $2.5 \pm 0.7\%$ ; Fig. 5). Systemic application of SB203580 before and during the preconditioning protocol (group VII) did not influence the IS limitation mediated by ischemic preconditioning ( $3.2 \pm 0.5\%$  for SB systemic; Fig. 5). Also in this case, IS was significantly lower than in the control group 2 (group IV). These results show that the infusion (local or systemic) of SB before and during ischemic preconditioning did not influence the IS limitation mediated by ischemic preconditioning. The infusion of KHB/DMSO before and during the preconditioning protocol did not influence the effect of ischemic preconditioning (Fig. 5).

#### Effect of SB203580 on p38-MAPK activities

The p38-MAPK activity and the phosphorylation state of this enzyme were investigated during index ischemia that followed the systemic infusion of SB or of the solvent. The ventricular drill biopsies were taken from the ischemic and nonischemic regions at time points described in the experimental protocol VIII (Fig. 1). Using an antibody that reacts specifically with dual-phosphorylated p38-MAPK (Thr180/Tyr182), we investigated the content of phosphorylated p38-MAPK after systemic infusion. In both SB and KHB infusion, we found a significant increase of phospho-p38-MAPK during ischemia (Fig. 6A and B), with a maximum reached at 20 min of ischemia and without significant differences between KHB- and SB-treated tissue. Only at 30 and 45 min of ischemia did SB significantly reduce the content of phospho-p38-MAPK. With an antibody that reacts

specifically with the phosphorylated form of SAPK/JNKs, we did not detect significant changes in phosphorylation of these kinases during ischemia after SB or KHB treatment (Fig. 6C). Western blot assay with a specific p38-MAPK antibody showed that there were no significant changes in p38-MAPK abundance when cytosolic fractions from untreated, KHB-, and SB-treated tissue were compared (Fig. 7A and B). Some decrease in content of p38-MAPK after SB infusion was observed after 45 min of ischemia, but this difference was not statistically significant. By means of in-gel phosphorylation of a specific p38-MAPK substrate (GST-MAPKAPK-2<sub>46-400</sub>), we investigated the effect of SB on p38-MAPK activity. We found that systemic infusion of SB for 10 min before index ischemia did not significantly change the p38-MAPK activities when compared with KHB (DMSO) infusion  $\leq 20$  min of ischemia (Fig. 8A and B). Only at 30 and 45 min of ischemia did SB significantly reduce the activity of p38-MAPK. The influence of SB on p38-MAPK activities during ischemia correlates with the observed time course of p38-MAPK phosphorylation (Fig. 6B). SB is an inhibitor that directly and reversibly influences the p38-MAPK. However, SB does not change the phosphorylation state of p38-MAPK itself (a factor important for activation of this enzyme), and after washout of the inhibitor by homogenization and buffer washes, the p38-MAPK is reactivated. For this reason, we investigated the p38-MAPK activities also when SB was present during the whole experimental procedure (especially by in-gel phosphorylation). In this case, we observed significant reduction of p38-MAPK activities (reduced phosphorylation of MAPKAPK-2) in the presence of SB (Fig. 8A and C).

#### Effect of SB203580 on the phosphorylation of ATF-2

To determine the in vivo effect of SB on p38-MAPK activities, we determined also the in vivo phosphorylation of activating transcription factor-2 (ATF-2). This transcription factor serves as an endogenous substrate for p38-MAPK, and we investigated its phosphorylation af-

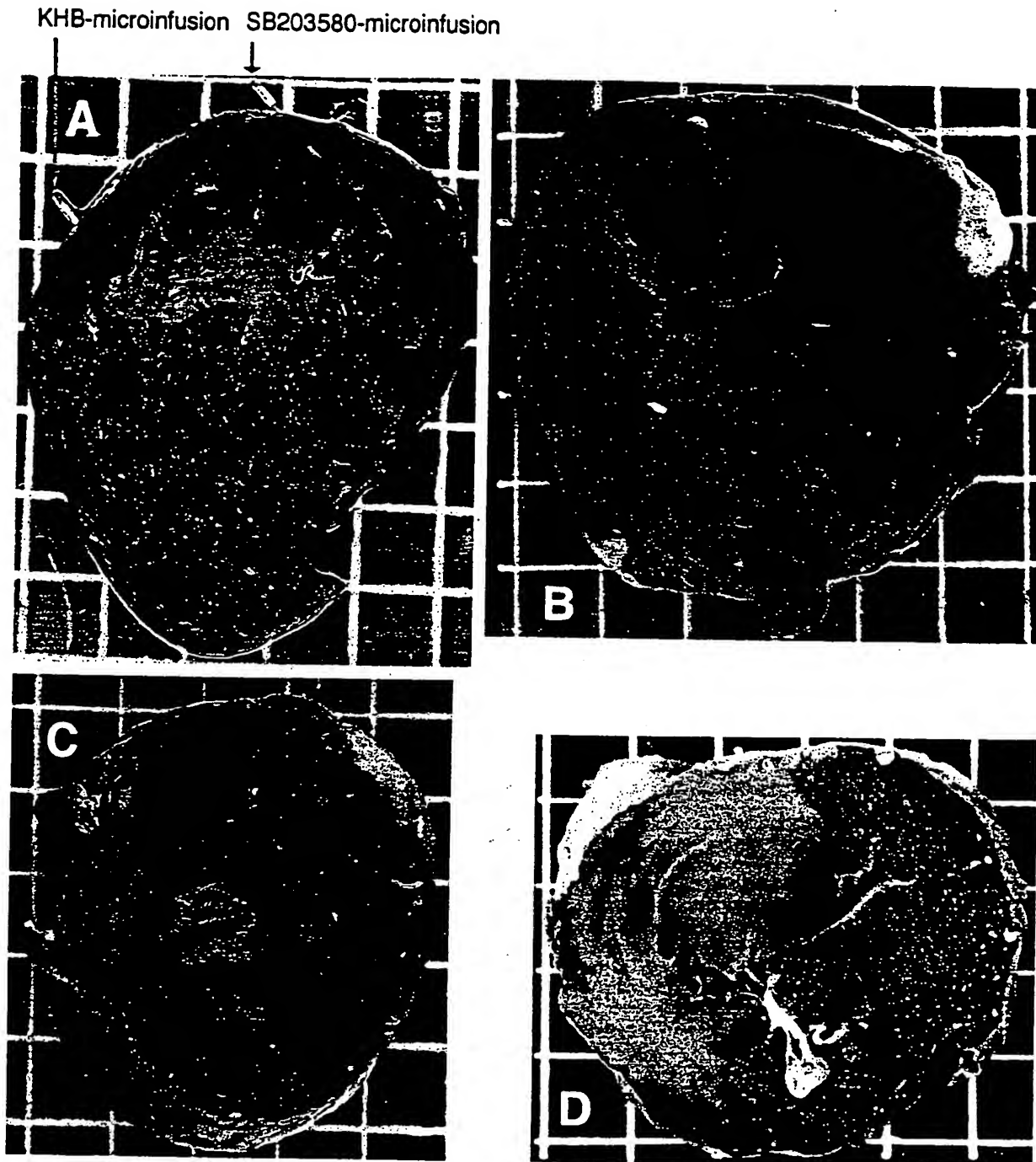


FIG. 4. Intramyocardial infusion of SB203580 (group VI; A) and systemic infusion of SB203580 (group VII; B) before and during ischemic preconditioning followed by index ischemia of 60-min occlusion and reperfusion of 60 min. A: Microinfusion of KHB (needle on the left) and SB203580 (40 nM, needle on the right). B: Systemic infusion of SB203580 (5 mg/animal). C: Preconditioning protocol (two cycles of 10-min ischemia and 10-min reperfusion; group V) followed by index ischemia of 40-min occlusion and reperfusion (60 min). D: Index ischemia for 40-min occlusion followed by reperfusion (60 min, group IV, control group 2).

ter systemic infusion of SB (or KHB as negative control) and during the following ischemia (group VIII; Fig. 1). We found that the presence of SB significantly inhibited the ischemia-induced phosphorylation of ATF-2 (Fig. 9). The content of phospho-ATF-2 was decreased after infusion of SB (compared with KHB control). In negative controls (KHB infusion), we observed during ischemia

increased phosphorylation of ATF-2 (maximum at 20 min of ischemia), but the presence of SB prevented the ischemia-induced p38-MAPK-mediated phosphorylation of ATF-2. Western blot assays with a specific ATF-2 antibody showed that there were no significant changes in ATF-2 abundance when nuclear fractions from untreated, KHB-, and SB-treated tissue were com-



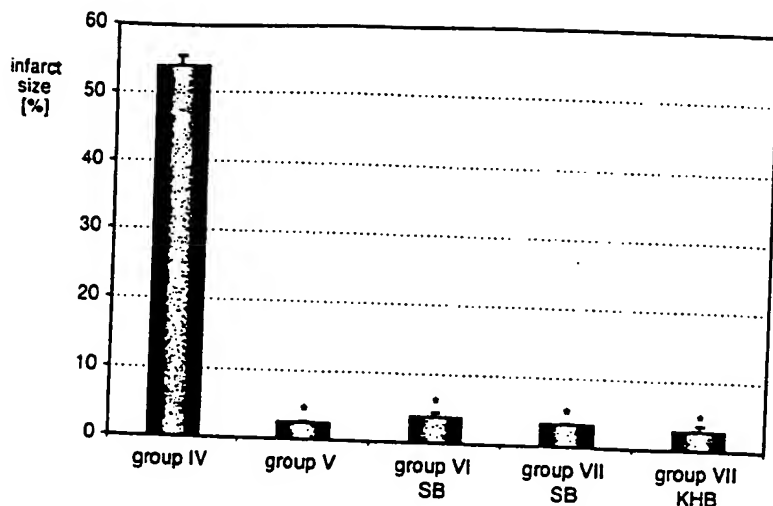


FIG. 5. Effect of local (group VI) and systemic (group VII) infusion of SB203580 on the cardioprotection induced by ischemic preconditioning. Group IV: Effect of KHB/DMSO infusion on infarct size after ischemic preconditioning. Values are expressed as percentage of the area at risk of infarction. Group IV is control group 2; group V underwent the ischemic preconditioning protocol. Each bar represents the mean  $\pm$  SEM. \* $p < 0.001$  (vs. group IV).

pared. This observation proves that the changes of phospho-ATF-2 reflect the different degree of phosphorylation of this transcription factor.

### DISCUSSION

The most important findings are our observations that (a) the inhibition of p38-MAPKinase pathway with the specific inhibitor SB before and during ischemia protects the myocardium against ischemic cell death, and (b) the systemic or local application of SB before and during the

ischemic preconditioning (IP) protocol did not influence the IP-mediated cardioprotection. We have previously reported that ischemia increased p38-MAPK activity, that reperfusion downregulated, and that repeated brief ischemia further downregulated its activity (4). We showed also the protective effect of p38-MAPK inhibition during ischemia (7). These results suggested an inverse correlation between p38-MAPK activation and survival (i.e., low p38-MAPK activity correlated well with an improved chance of survival and vice versa). Because uninterrupted ischemia is the fastest way to cell death,

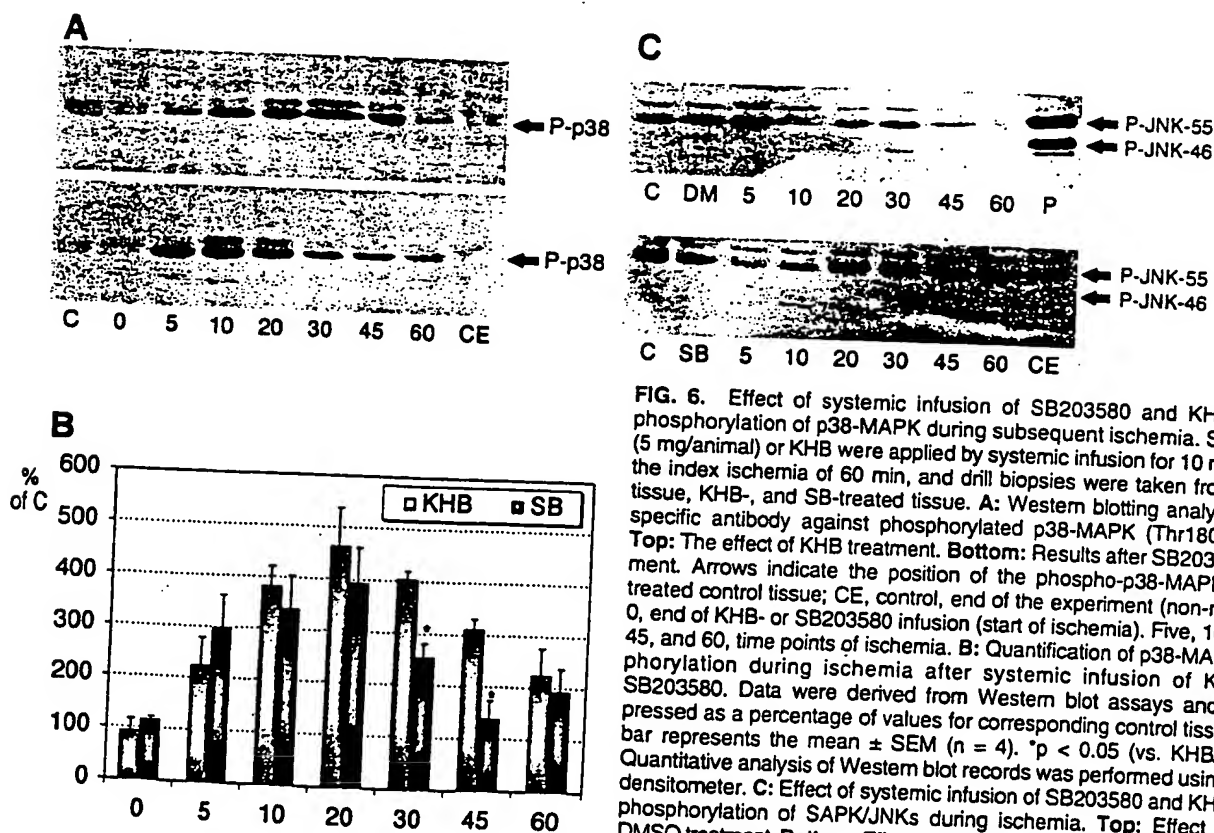


FIG. 6. Effect of systemic infusion of SB203580 and KHB on the phosphorylation of p38-MAPK during subsequent ischemia. SB203580 (5 mg/animal) or KHB were applied by systemic infusion for 10 min before the index ischemia of 60 min, and drill biopsies were taken from control tissue, KHB-, and SB-treated tissue. A: Western blotting analysis with a specific antibody against phosphorylated p38-MAPK (Thr180/Tyr182). Top: The effect of KHB treatment. Bottom: Results after SB203580 treatment. Arrows indicate the position of the phospho-p38-MAPK. C, untreated control tissue; CE, control, end of the experiment (non-risk area); 0, end of KHB- or SB203580 infusion (start of ischemia). Five, 10, 20, 30, 45, and 60, time points of ischemia. B: Quantification of p38-MAPK phosphorylation during ischemia after systemic infusion of KHB and SB203580. Data were derived from Western blot assays and are expressed as a percentage of values for corresponding control tissue. Each bar represents the mean  $\pm$  SEM ( $n = 4$ ). \* $p < 0.05$  (vs. KHB/DMSO). Quantitative analysis of Western blot records was performed using a laser densitometer. C: Effect of systemic infusion of SB203580 and KHB on the phosphorylation of SAPK/JNKs during ischemia. Top: Effect of KHB/DMSO treatment. Bottom: Effect of SB203580 treatment. P, positive control.



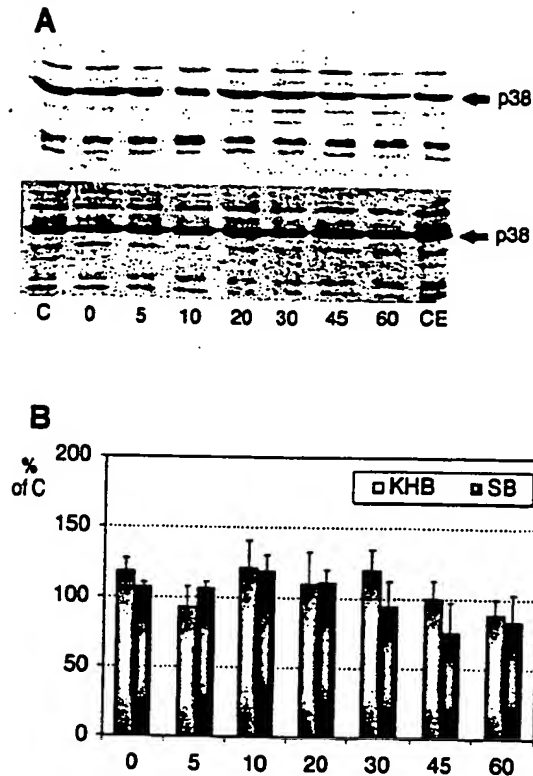


FIG. 7. Effect of systemic infusion of SB203580 (5 mg/animal) and KHB on the content of p38-MAPK during ischemia. A: Western blot analysis with a specific antibody against p38-MAPK. Top: Results after KHB treatment. Bottom: After SB203580. Arrows indicate the position of the p38-MAPK. C, untreated control tissue; CE, control, end of experiment (non-risk area); 0, end of KHB or SB203580 infusion (start of ischemia); 5, 10, 20, 30, 45, and 60, time points of ischemia. B: Quantification of p38-MAPK content during ischemia after systemic infusion of KHB and SB203580. Each bar represents the mean  $\pm$  SEM ( $n = 4$ ).

we hypothesized that p38-MAPK stimulation is the cause for accelerated or premature cell death. This hypothesis was tested by systemic and by local infusion of a p38-MAPK inhibitor, the pyridinyl imidazole compound SB. The infusion of SB markedly increased the tolerance to ischemia, especially when infused locally into the myocardium, a useful method for the study of tool drugs that are either too costly or too toxic for systemic use (8,9). SB also significantly reduced IS after systemic i.v. injection. When care was taken that the SB also was present during the phosphorylation step by in-gel phosphorylation, it exhibited a powerful inhibitory effect on p38-MAPK. ATF-2 is a transcription factor that serves in vivo as a substrate for the p38-MAPK cascade. We observed increased phosphorylation of this transcription factor during ischemia, but the phosphorylation was significantly reduced as a consequence of p38-MAPK inhibition by SB. It was reported that SB at high concentrations inhibits 52- and 54-kDa SAPK/JNK but not the 46-kDa JNK-1 (10). Ten micromolar SB completely inhibited the 54-kDa SAPK/JNK and partially inhibited the activity of 52-kDa SAPK/JNK. The  $IC_{50}$  for inhibition of p38-MAPK-mediated stimulation of

MAPKAPK-2 observed in the cited study was  $\sim 70$  nM and 3–10  $\mu$ M for total SAPK/JNK activity. The concentration of SB used in our study was 40 nM. The concentrations that would inhibit also SAPK/JNKs were therefore not reached. Moreover, we and others found that the activities of SAPK/JNKs are unaffected by ischemia, and activation occurs only during reperfusion (4,11,12). In this study we did not observe stimulation (increased phosphorylation) of SAPK/JNKs activities during ischemia. For this reason, the SAPK/JNKs are unlikely contributors in the increased phosphorylation of ATF-2 during ischemia. Our findings strongly suggest that p38-

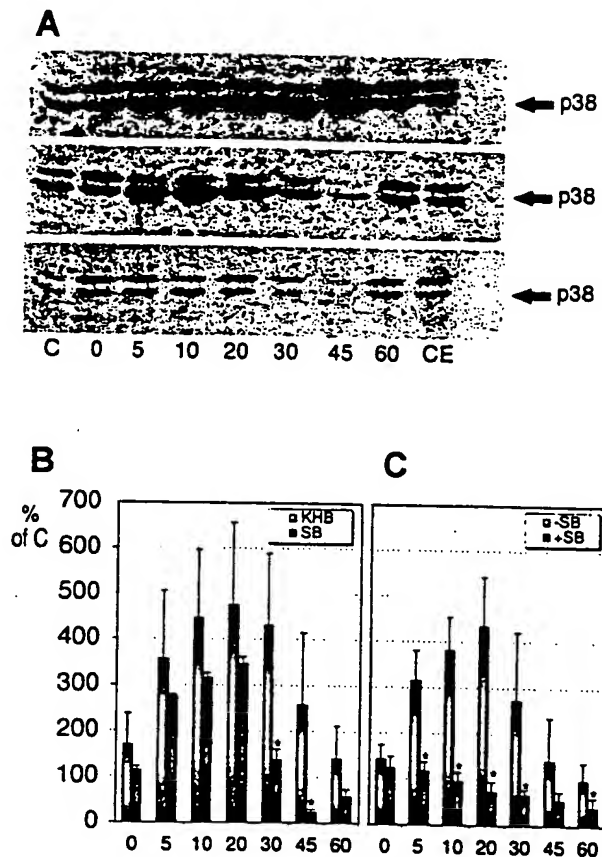


FIG. 8. Effect of systemic infusion of SB203580 (5 mg/animal) and KHB on the activity of p38-MAPK during ischemia. A: The in-gel phosphorylation of MAPKAPK-2 was performed as described under Materials and Methods. Top: Gel after KHB infusion. Middle: Gel after SB203580 infusion. Bottom: In-gel phosphorylation of MAPKAPK-2 in the presence of SB203580. The arrows indicate the positions of p38-MAPK. C, control tissue; CE, control, end of experiment (non-risk area); 0, end of KHB or SB203580 infusion (start of ischemia); 5, 10, 20, 30, 45, and 60, time points of ischemia. B: Quantification of p38-MAPK activation during ischemia after systemic infusion of KHB and SB203580. Data were derived from in-gel kinase assays and are expressed as a percentage of value for corresponding control-untreated tissue. Each bar represents the mean  $\pm$  SEM ( $n = 4$ ). \* $p < 0.05$  (vs. KHB/DMSO). C: Quantification of p38-MAPK activities from gels when SB203580 was absent (-) or present (+) during in-gel phosphorylation. Each bar represents the mean  $\pm$  SEM. \* $p < 0.05$  (vs. SB). Quantitative gel analysis was performed using Phosphorimager SF (Molecular Dynamics).

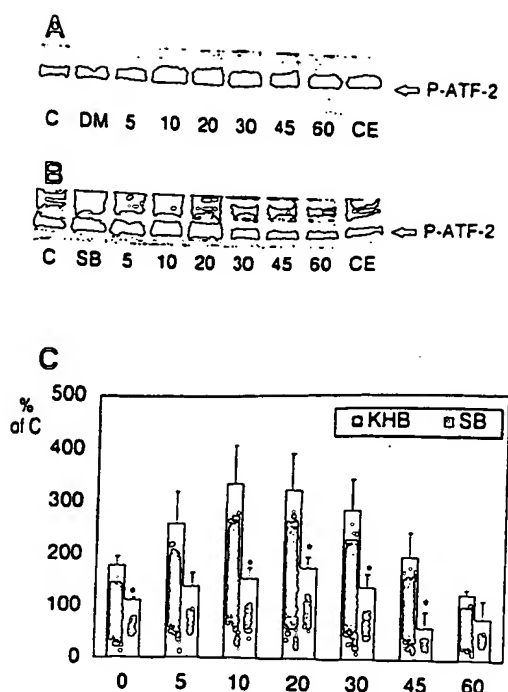


FIG. 9. Western blotting analysis with a specific antibody against phospho-ATF-2. A: After KHB treatment. B: After SB203580 treatment. Arrows indicate the position of the phospho-ATF-2. C, untreated control tissue; CE, control, end of experiment (non-risk area); DM, end of KHB infusion; SB, end of SB203580 infusion (start of ischemia); 5, 10, 20, 30, 45, and 60, time points of ischemia. C: Quantification of content of phosphorylated ATF-2 during ischemia after systemic infusion of KHB and SB203580. Data were derived from Western blot assays and are expressed as a percentage of values for corresponding control tissue. Each bar represents the mean  $\pm$  SEM ( $n = 4$ ). \* $p < 0.05$  (vs. KHB/DMSO). Quantitative analysis of Western blot records was performed using a laser densitometer.

MAPK is part of a pathway accelerating cell death. These findings are in contrast to those of others who found, in the rabbit, that p38-MAPK is the pathway favoring survival. In the study of Weinbrenner et al. (13), it was shown that ischemia caused no increased phosphorylation of the tyrosine residue of p38-MAPK in the rabbit heart, and the increased phosphorylation occurred only when the heart was preconditioned. However, our previous findings with regard to p38-MAPK activation (increased phosphorylation) during ischemia (4) in pig myocardium are in agreement with Bogoyevitch et al. (11), who found in isolated rat heart that the p38-MAPK activity was strongly activated by ischemia alone. In contrast to our findings, this activation was further increased during reperfusion. These somewhat divergent results strongly suggest the existence of species differences. Our results are in support of those by Ma et al. (14), who showed that administering SB before ischemia and during reperfusion completely inhibited p38-MAPK activation and exerted a beneficial effect on the recovery of myocardial function and reduced the incidence of apoptosis. The study of Mackay and Mochly-Rosen (15) demonstrated a protective effect of SB against extended isch-

emia in cultured neonatal rat cardiomyocytes. These cited studies, which all supported the view of a negative survival value of p38-MAPK in ischemia, are in contrast to the earlier results by Weinbrenner et al. (13), who observed that SB completely abolished the protection from ischemic preconditioning in isolated cardiomyocytes and suggested a positive role of p38-MAPK in preconditioning. Nagarkatti and Sha'afi (16) also found that the protective effect of preconditioning stimuli was abolished in the presence of SB but not in the presence of MEK inhibitor PD98059 in the rat myoblast cell line H9C2. In contrast to the results of these studies, we found that both systemic and local applications of SB before and during brief coronary occlusions did not influence the protection by ischemic preconditioning. Moreover, in contrast to the results of Nagarkatti and Sha'afi (16), we recently found that the MEK inhibitor PD98059 abolished the protective effect of preconditioning (17). Paradoxically the study of Nagarkatti and Sha'afi reported a protective effect of SB203580 when present during the lethal ischemic stress. We found previously that ischemia in our experimental model stimulated the p38-MAPK activity, but attenuation occurred during repeated ischemia (4). It is of interest that Nagarkatti and Sha'afi (16) support our finding that ischemic stress activates p38-MAPK and that preconditioning decreased activation of p38-MAPK in response to repeated ischemia. Our results with the p38-MAPK inhibitor and SAPK/JNK activators (7,18,19) support those of a recent report by Wang et al. (20), who studied the role played by MKK7 and found that p38-MAPKs promote cell death in cultured cardiac myocytes and that the JNKs were important for hypertrophy but also favored survival. It was shown that in isolated perfused rat hearts, ischemic stress was associated with nuclear translocation and activation of nuclear factor  $\kappa$ B (NF $\kappa$ B), which was significantly blocked by genistein and SB (21). It was also reported that the activation of p38-MAPK and of NF $\kappa$ B leads to tumor necrosis factor (TNF) production (22), which contributes to postischemic myocardial dysfunction. In isolated perfused rat hearts, it was found that p38-MAPK inhibition or treatment with TNF-binding protein decreased myocardial TNF production, cardiomyocyte death, and myocardial dysfunction (23). These observations also suggest the negative survival value of p38-MAPK during ischemia and show that inhibition of p38-MAPK increases the ischemic tolerance.

In this study and also in our previous experiments, we observed two bands (protein kinases) in the range of 38–45 kDa that use MAPKAP-2 as a substrate *in vitro*. We investigated the specificity of the reaction for p38-MAPK by immunoprecipitation with a p38-MAPK polyclonal antibody (C-20). The antibody is specific for p38-MAPK and is not cross-reactive with p38-MAPK-beta. The same antibody was used for detection of p38-MAPK content presented in this study (see Fig. 7A). We found that the antibody reacted very strongly with a 38-kDa protein of molecular mass of (p38-MAPK) and when the immunoprecipitate was tested by in-gel phosphorylation

of MAPKAPK-2, we found activity only in the range of 38 kDa. We cannot exclude the possibility that the upper band of 45 kDa represents some isoform of p38-MAPK, but our results show that SB inhibits preferentially the activity of the lower (38-kDa) band. It is known that the p38-MAPK exists in at least six isoforms (two alternative spliced isoforms  $\alpha$  and  $\beta$  and isoforms  $\tau$  and  $\delta$ ). These p38-MAPKs differ in their sensitivity to stimulation, inhibitor sensitivity, and also substrate specificity. We cannot exclude the possibility that more than one isoform of p38-MAPK is activated during myocardial ischemia. However, PC12 cells showed a selective activation of p38-MAPK- $\alpha$  and p38-MAPK- $\gamma$  by hypoxia (24). Hypoxia had no effect on the activity of the  $\beta$  and  $\delta$  isoforms. Our results obtained with SB in vitro (phosphorylation step of in-gel assay; Fig. 8A and C) show that SB fully inhibited the ischemia-induced p38-MAPK activity. It has been described that the  $\gamma$  and  $\delta$  isoforms are resistant to inhibition by SB (25,26). This would suggest that these two p38-MAPK isoforms ( $\gamma$  and  $\delta$ ) are not involved in the effects of SB during ischemia and in mechanisms leading to ischemic death.

In conclusion, we provide detailed information about the detrimental effect of p38-MAPK activation during ischemia, which can be inhibited by SB. We have provided further evidence for our hypothesis that ischemia/reperfusion activates different signaling cascades with opposing effects on survival, of which the ERKs and the SAPK/JNKs favor survival, and the p38-MAPKs accelerate cell death. The development of future treatment strategies for ischemic syndromes may find these observations useful.

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